INHIBITORY EFFECTS OF KETOCONAZOLE ON THE OXIDATION OF LINOLEIC ACID MICELLES, PHOSPHOLIPID LIPOSOMES, AND HUMAN LOW DENSITY LIPOPROTEIN (H-LDL)

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Master of Arts

by

Melissa E. Munroe

July 1993

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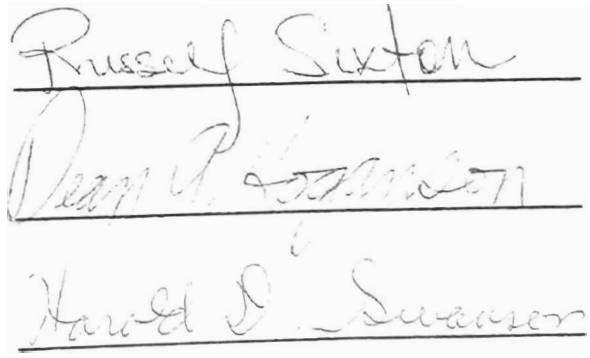
Approved by Committee



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An abstract of a Thesis by

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Soybean lipoxygenase (SLO) (500-5000 units/ml), $Fe(III)Cl_3$/ascorbate (5-100μM) and hematin (5-100μM) induced oxidative modification of lipids present in micelles of linoleic acid (0.5 mM), liposomes prepared from phospholipid extracts of bovine brain (2.5 mg/ml), and h-LDL (0.5 mg/ml). Oxidation of the lipid substrates was determined by the formation of thiobarbituric acid reactive substances and conjugated dienes. Free iron ($Fe(III)Cl_3$/ascorbate) was the strongest initiator, followed by hematin and SLO. Ketoconazole (KC) attenuated lipid oxidation in a concentration dependent manner at pharmacologically relevant levels (25-100μM) in all three substrates. Pretreatment of liposomes and h-LDL with KC produced greater attenuation of lipid oxidation by the various initiators in comparison to free KC.

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INTRODUCTION AND REVIEW OF THE LITERATURE

Experimental and clinical studies have shown that elevated levels of low-density lipoprotein (LDL) in plasma significantly contribute to premature atherosclerosis. Atherosclerosis, one of the most prevalent causes of morbidity and mortality in man, is due to overaccumulation of lipids, mostly free and esterified cholesterol, in the form of LDL entering the arterial intima, the site of atherogenesis. The failure of the cholesterol to retreat back from the intima into the circulation on high density lipoprotein (HDL) particles results in the eventual formation of fatty streaks. Affected sites in the intima accumulate cholesterol early in the atherosclerotic process in structures known as atheromas, formed as consequence of the disturbed cholesterol flow through the intima caused by an imbalance between influx and efflux of lipoproteins (Vance and Vance 1991).

These atheromas are made up of foamy droplets that create a fatty-streak. The earliest recognized gross lesion in atherosclerosis is the fatty streak, characterized by an accumulation of "foam" cells loaded with cholesterol esters just beneath the endothelium. Most foam cells arise from circulating monocytes/macrophages that have taken up residence beneath the vascular endothelium, although some are derived from medial smooth-muscle cells (Steinberg et al. 1989). The trapping of foam cells in the innermost layer of the artery wall in addition to the release of growth factors and chemoattractants is thought to initiate and perpetuate proliferation of smooth muscle cells and deposition of components of connective tissue that form the advanced atherosclerotic plaque (Sparrow et al. 1988).

Circulating monocytes/macrophages contain "scavenger receptors" which are specific sites for the recognition of LDL. Significantly, the intimal monocyte-macrophages are transformed into the characteristic lipid-filled foam cells found in human atherosclerotic lesions (Vance and Vance 1991). However, before the "scavenger receptors" can take up the LDL and promote the formation of foam cells, the LDL must be oxidatively modified

(Carew 1989). Isolated macrophages have few LDL receptors and fail to take up native LDL; however, LDL chemically modified in vitro into acetylated LDL or oxidized LDL, is avidly bound and taken up by macrophages (Vance and Vance 1991). Oxidative modification of the lipoprotein has been shown to render it chemotactic to macrophages and cytotoxic to endothelial and arterial smooth muscle cells. Additional macrophages are recruited and subsequently circulated to the subendothelial space and within the arterial walls where further modification occurs to form advanced lesions or "plaques".

There are several possible pathways of oxidative modification (Figure 1). These pathways can originate from two sources: within the cell itself and from the surrounding media or cytoplasm. Cells may induce oxidation in any of three pathways. In one, the lipids in the cell are oxidized first, after which they transfer into the LDL in the medium and initiate chain reactions that oxidize the lipids in the LDL extensively. A second pathway could involve the direct oxidation of LDL lipids during LDL-cell contact. A third pathway postulates the generation of superoxide anion (or other activated oxygen) within the cell, followed by release into the medium, where the oxidation of LDL lipids then occurs (Steinberg et al. 1989). Cell-mediated oxidation can come from two sources: endothelial cells or smooth muscle cells within the arterial intima. During its incubation with cells, the LDL particle undergoes a large number of structural changes that alter its metabolism in important ways.

All these changes depend on a common initiating step--the peroxidation of polyunsaturated fatty acids in the LDL lipids (Steinberg et al. 1989). It has been proposed that the modification of LDL in endothelial cells involves radical-initiated LDL peroxidation (Steinbrecker 1988) Initiators of such modification may include ferritin (an iron containing structure), hydrolytic enzymes (involved in a number of metabolic processes), cytochrome P450 enzymes (involved in steroid production), or as additionally suggested by S. Parthasarathy et al. (1989), lipoxygenase. Endothelial cells may initiate lipid peroxidation,

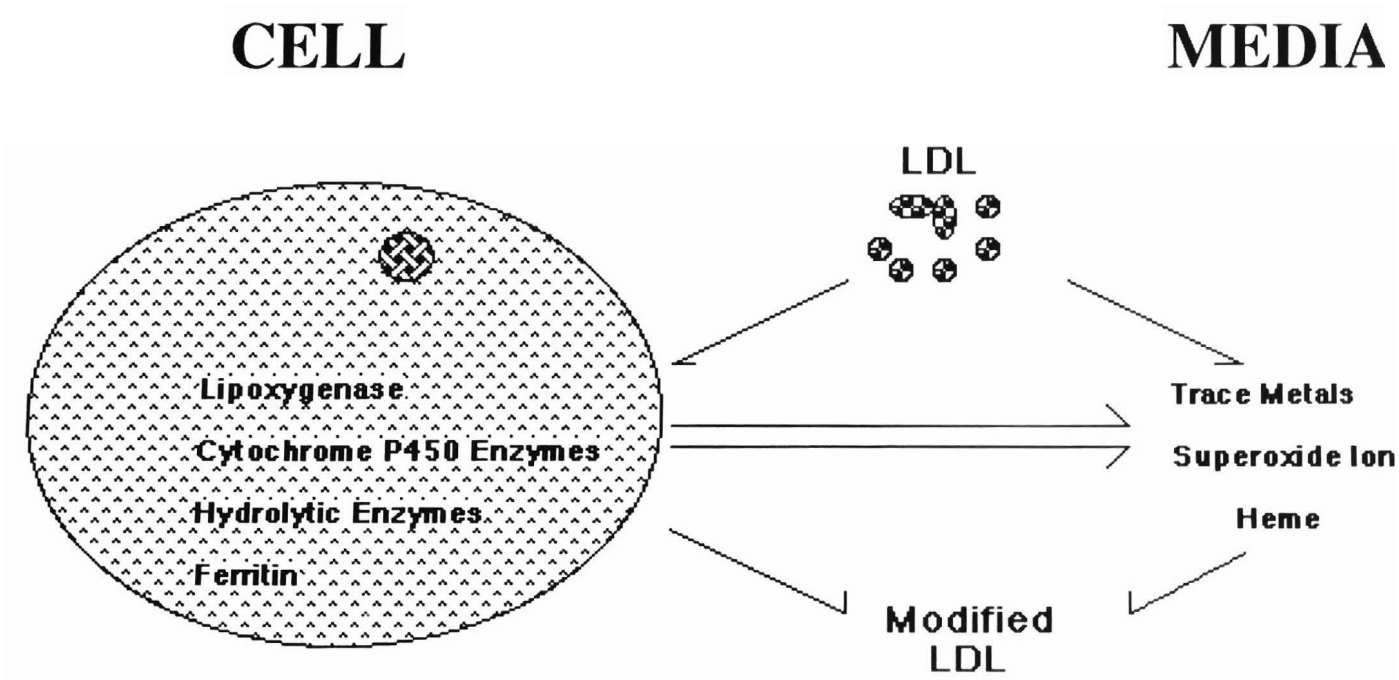


Figure 1. Before LDL can be taken up by macrophages to form foam cells, it must first be oxidatively modified. This can occur within the cell by various components (e.g. lipoxygenase) or outside the cell in the media by initiators such as trace metals, superoxide ions (a result of previous oxidation within the cell), or heme.

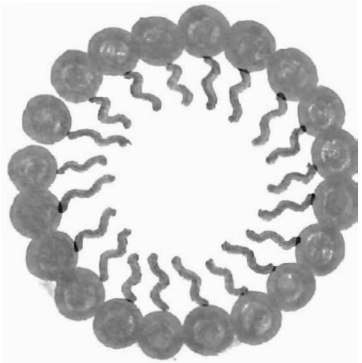
as mediated by superoxide ion (or by other substances that can also transfer electrons to transition metal ions. The superoxide ion appears to be the significant mediator, as in the enzyme superoxide dismutase, which catalyzes the formation of hydrogen peroxide from superoxide, is consistently inhibitory (26).

According to Morel et al. (1984), events vascular smooth muscle cells are similar to those in endothelial cells. Heinecke (1988) clarifies this assumption by indicating that extracellular superoxide generation and LDL modification by arterial smooth muscle cells in culture are dependent on the presence of sulfur containing amino acids such as L-cystine in the incubation medium. Furthermore, LDL modification, but not superoxide generation, also requires the presence of micromolar concentrations of transition metals such as Cu(II) or Fe(III). It has been additionally proposed by Cathcart et al. (1988) that monocytes/macrophages can further modify LDL after trapping it into the intima in similar fashion to that of endothelial and smooth muscle cells.

Many pathways of noncellular modification of LDL have been suggested. Sparrow et al. (1988) have proposed that oxidation of LDL can be mimicked by using soybean lipoxygenase (SLO), a 15-lipoxygenase. It appears that the 15-lipoxygenase is the most prevalent type of lipoxygenase that can catalyze the oxygenation of unsaturated fatty acids attached to phospholipids to render them chemotactic to monocytes and cytotoxic to all cells (Henriksen 1993). However, the optimal substrate for SLO is not LDL, but rather linoleic acid micelles (Ford-Hutchinson 1991). Another substrate which has been successfully applied to peroxidation studies and provides an ideal model system as an oxidizable substrate and an organized lipid membrane bilayer is ox-brain phospholipid liposomes (Carew 1989) Figure 2 illustrates the basic structures of these three substrates used in this study.

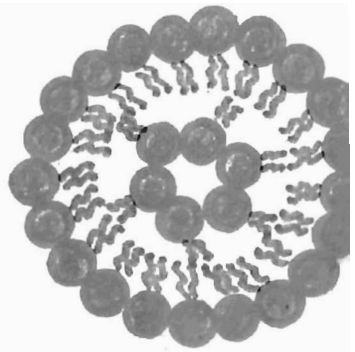
Oxidation of lipid substrates may also involve iron (III) chloride/ascorbate, a free iron moiety, and hematin, containing a heme-like iron. Iron (III) chloride/ascorbate has been

SUBSTRATES

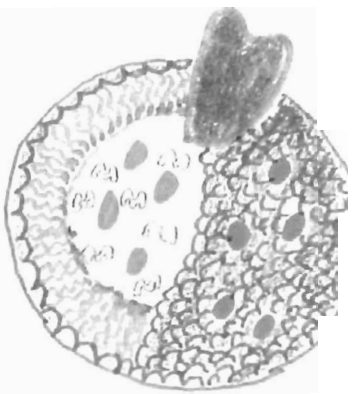


Linoleic Acid Micelles

Hydrophilic Head
Hydrophobic Chains



Ox Brain Phospholipid
Liposomes



Human LDL Particle

Free (Unesterified) Cholesterol
Cholesterol Esters
Triacylglycerols
ApoB-100 protein
Phospholipid monolayer

Figure 2. Structures of substrates used in comparative study (oxidative modification of lipids by soybean lipoxygenase [SLO], iron [III] chloride/ascorbate, and hematin and subsequent attenuation of oxidation by ketoconazole).

shown to be an oxidant of phospholipid liposomes (Wiseman et al., 1991). Hematin has been shown to oxidize linoleic acid, launching further oxidation from the auto-oxidation already occurring in the micelle (Dix and Marnett 1985). It has been suggested that hemin, an analog of hematin with a heme-like iron, is capable of modifying LDL (Bella et al. 1991). These iron moieties can be found outside of the cell and would work well to complement the active non-heme iron moiety of lipoxygenase. It has been proposed that in linoleic acid, the lipoxygenase iron goes from the iron(III) to the iron(II) state as linoleic acid is oxidized to a pentadienyl radical intermediate (Folcik and Cathcart 1993).

Interventions that block oxidative modification of LDL are under intensive investigation. Of interest is the inhibition of lipid oxidation by antioxidants and metal chelating agents. The free-radical quenching antioxidants such as butylated hydroxytoluene (BHT) and vitamin E have been shown to inhibit LDL oxidation. The drug probucol, similar in structure to BHT, is showing some promise as an antioxidant as well (Steinberg and Witelum 1990). Agents such as ketoconazole that inhibit cytochrome P-450 enzymes are of additional interest as inhibitors of enzyme-induced oxidation. Ketoconazole is currently marketed as an antifungal drug and is a known cytochrome P-450 enzyme inhibitor . It has been recently reported by Wiseman et al. (1991) that ketoconazole may be considered an antioxidant in that it is capable of inhibiting the oxidation of liposomes when under oxidative attack by iron(III) chloride/ascorbate. In clinical investigations, it has been further demonstrated by Gylling et al. (1993) that ketoconazole greatly reduces the level of cholesterol precursors and low density lipoprotein when taken orally (400 mg/day for 5 weeks).

The purpose of this study is to determine the antioxidant nature of ketoconazole in conjunction with SLO, iron (III) chloride/ascorbate, and hematin. The lipid substrates to be used are linoleic acid micelles, phospholipid liposomes, and LDL. While this drug may act as a chelating agent to prevent iron moieties from attacking lipids and therefore inhibit

oxidative modification, it is also possible that ketoconazole acts to protect the lipid membrane shell from oxidative attack either by surrounding the lipid particle or incorporating itself directly into the membrane.

MATERIALS AND METHODS

Linoleic Acid Preparation (Micelles)

Linoleic acid (50µl), obtained from Sigma Chemical Company (St. Louis, Mo.), was added to 50µl of 95% ethanol and vortexed in a microfuge tube. The emulsion (100µl) was added to 49.9ml distilled water (10mM linoleic acid), vortexed further, and diluted to a concentration of 0.5mM in chelated (using Chelex^{100} resin from Bio-rad) phosphate buffered saline (PBS), pH 7.4 (containing 140mM NaCl, 2.7mM KCl, 16mM Na_2HPO_4, and 2.9mM KH_2PO_4) or in bis-tris propane buffer (BTP), 0.2M for pH studies.

Ox-Brain Phospholipid Preparation (Liposomes)

Liposomes were prepared according to the method of Helen Wiseman et al. (1991) and Gutteridge (1977). Ox-brain phospholipid (fraction V) was obtained from Sigma Chemical Company (St. Louis, Mo.) and dissolved in chloroform to give a final concentration of 5 mg/ml. The chloroform was evaporated under a stream of nitrogen at room temperature. The residue was then resuspended in chelated PBS at the original volume and vortexed for 5 minutes in the presence of glass beads. The resulting liposomes were left to stand for one hour at 4°C prior to use.

Human Low-density Lipoprotein (LDL) Preparation

LDL was isolated from pooled normal human plasma (obtained from the Blood Center of Central Iowa) using density gradient ultracentrifugation according to the protocol of Radding and Steinberg (1960). The isolated LDL was put under nitrogen and stored in the dark at 4°C.

Protein/phospholipid determination of LDL

The Bicinchoninic acid (BCA) protein assay kit was obtained from Sigma and used for protein determination of LDL. A standard curve was prepared using various concentrations of bovine serum albumin, Fraction V (BSA) and working reagent in a 200µl reaction mixture on a microtiter plate. LDL reaction mixtures were similarly prepared; all the reaction mixtures were incubated at 37°C for 30 minutes. An absorbance reading was taken at 562 nm. The protein content of the LDL sample was determined according to the standard curve. Phospholipid content was taken to be equal to protein content (Lehninger et al. 1993).

Soybean Lipoxygenase (SLO) Preparation

SLO was obtained from Sigma Chemical Company (St. Louis, Mo.) and stored at 10,000 units/ml in ice-cold borate buffer (0.2M), pH 9.0. One SLO unit will cause an increase in A_{234} of 0.001 per minute at pH 9.0 at 25°C, when linoleic acid was used as substrate. Reaction Volume = 3.0 ml (1 cm light path). The unit definition is in accordance to Sigma.

Iron (III) Chloride/Ascorbate and Hematin Preparation

Stock Iron (III) chloride and ascorbate solutions (1mM) were prepared in distilled water and distributed in 1:1 molar ratios in all reactions with lipid substrates. Stock solutions of hematin (1mM) were warmed in distilled water until the hematin was dissolved and subsequently aliquoted appropriately in all reactions with lipid substrates.

Ketoconazole (KC)

Ketoconazole was used in free form (0-100µM) in all three substrates and directly incorporated into liposomes and LDL. In liposomes, ketoconazole was dissolved in chloroform and incorporated into the phospholipid (in a mole:mole ratio) before nitrogen evaporation of the chloroform and subsequent liposome formation . In LDL, ketoconazole, dissolved in ethanol, was incubated at 37°C on a mole:mole ratio with phospholipid content

for 6 hours. The remaining free ketoconazole was then dialyzed away for 18 hours in chelated PBS (pH 7.4) at 4°C.

Thiobarbituric Acid Reactive Substances Assay (TBARS)

The reaction samples were analyzed for the amount of malondialdehyde (MDA) equivalents based on the TBARS assay as described by Morel et al. (1984). One ml of the reaction mixture was combined with 1 ml of 1.26% trichloroacetic acid (TCA) and 1 ml of 25% thiobarbituric acid (TBA). This reaction was heated for 45 minutes at 95°C. After cooling, the samples were read at an absorbance of 532 nm. An increase in absorbance readings in comparison to auto-oxidation indicates an increase in the oxidation of the lipid substrate. A standard curve was prepared using 1,1,4,4 tetramethoxypropane.

Conjugated Diene Formation

When linoleic acid is oxidized with SLO or hematin, fatty acid hydroperoxides are formed containing conjugated double bonds, showing a UV absorption maximum at 234 nm. The conjugated lipid hydroperoxides are labile intermediates and decompose to a great variety of products. When A_{234} measurements are taken directly after initiation of oxidation, the increase of the diene absorption is always preceded by a lag phase during which the 234-nm absorption remains constant. Thereafter, the 234-nm absorption rapidly increases (propagation period). It was during the propagation period that an accurate change in 234-nm absorption was determined and comparative amount of oxidation ascertained (Gebicki et al. 1991). Conjugated diene formation can be readily seen in linoleic acid oxidation and therefore monitoring UV absorbance values at 234 nm was ideal for monitoring the progress of oxidative modification. Oxidation was expressed in a change of A_{234}/minute.

Reaction Conditions for Oxidation of Lipid Substrates

When the thiobarbituric reactive substances assay (TBARS) was to be used, prepared linoleic acid micelles (0.5mM), ox-brain phospholipid liposomes (2.5 mg/ml), and LDL

(0.5 mg protein/ml), in PBS (pH 7.4) were oxidized at 37°C with SLO (500-5000 units), iron (III) chloride/ascorbate (5-100μM), and hematin (5-100μM). Subsequent data was normalized for comparison.

When conjugated diene formation was to be measured at an absorbance of 234 nm, linoleic acid micelles (0.5mM) were oxidized with SLO (1000 units) or hematin (5-50μM) at room temperature, and readings were taken immediately.

RESULTS

It has been previously shown that linoleic acid micelles (Ford-Hutchinson, 1991), phospholipid liposomes (Wiseman et al, 1991), and LDL (Cathcart, 1991) are all readily oxidizable substrates that can be monitored with the TBARS assay. At physiological temperature and pH, oxidation was achieved in all three substrates by all three initiators. As shown in figure 3, linoleic acid micelles, phospholipid liposomes, and LDL were treated with soybean lipoxygenase (SLO) (5000 units), iron (III) chloride/ascorbate (50μM, 1:1), and hematin (50μM). Data from these experiments were normalized for comparison. All underwent oxidation. Iron (III) chloride/ascorbate oxidized the three lipid substrates the most rapidly followed by hematin. SLO at pH 7.4 was much less effective. Linoleic acid was most readily oxidized, followed by LDL. Phospholipid was much slower to react.

As suggested by Ford-Hutchinson (1991), the optimal pH for SLO is not physiological (pH 7.4) but rather about pH 9. Figure 4 shows that while it was possible to achieve oxidative modification of linoleic acid (0.5 mM) by SLO at pH 7.4 (as compared to the auto-oxidation control), there was greater activity between pH 8.5 and 9.0. Figure 5 demonstrates that KC (in "free" form) was able to attenuate linoleic acid oxidation by SLO (1000 units) both at physiological and optimal pH ranges for the enzyme. At

Figure 3. Oxidation of lipids with SLO, iron (III)/ascorbate, and hematin. Linoleic acid micelles, phospholipid liposomes, and LDL were oxidized with the initiaors SLO, $FeCl_3$/ascorbate, and hematin at the indicated concentrations. The initiators were added to the lipid substrates in PBS, pH 7.4 at 37°C. The extent of oxidation was measured via the TBARS assay as described in Materials and Methods. Data points represent the mean values of triplicate samples with SE < 6%.

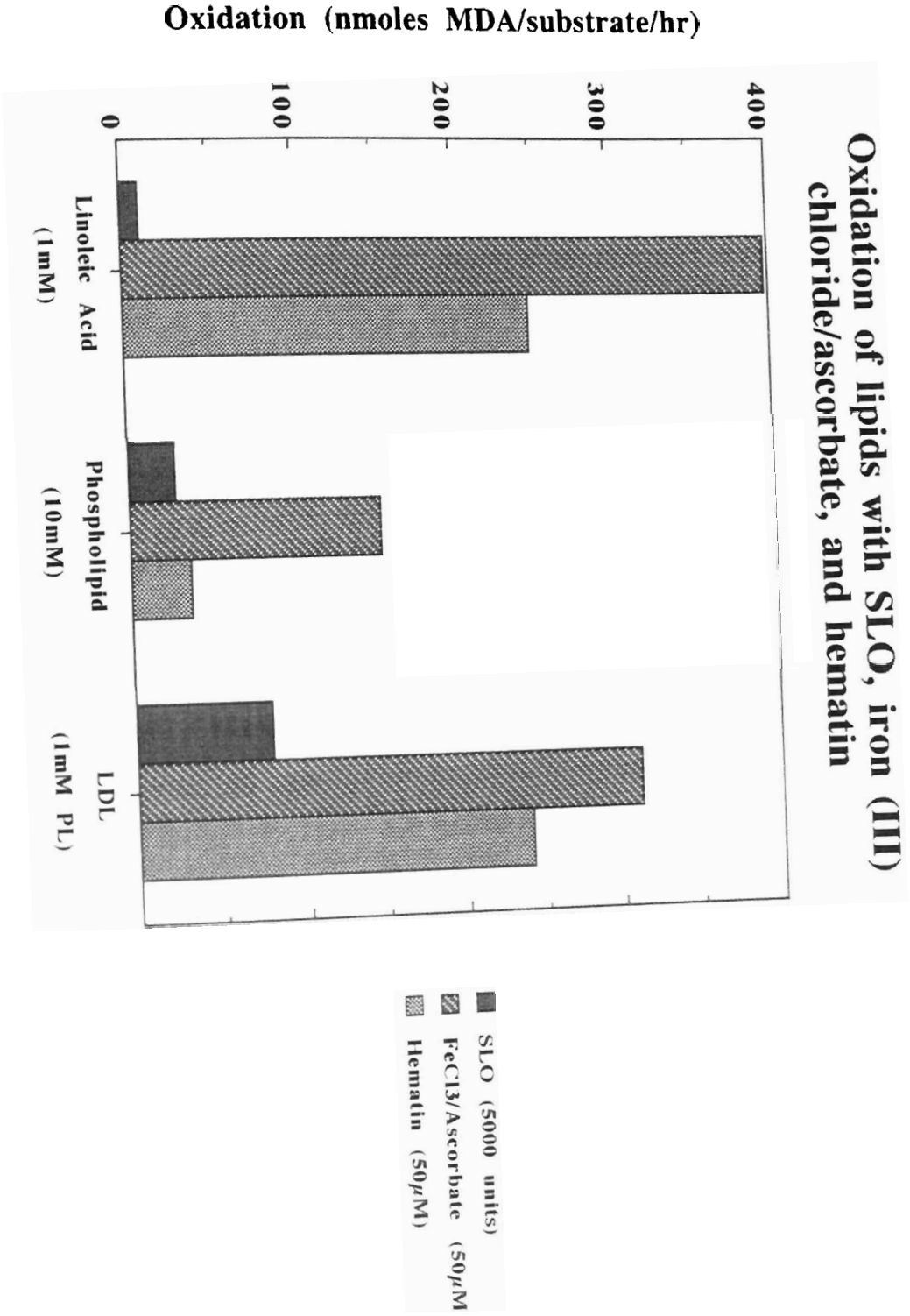
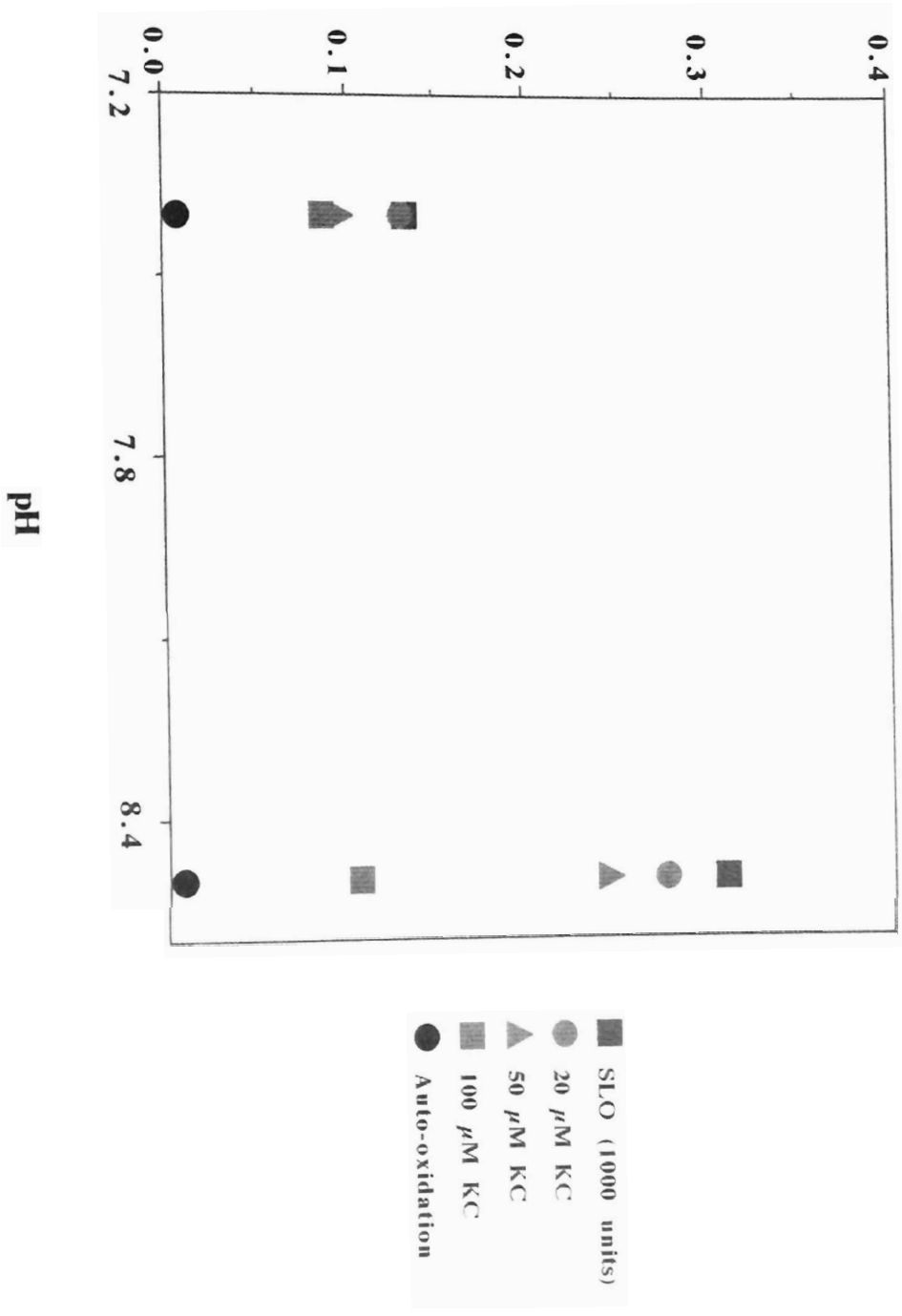

Oxidation of lipids with SLO, iron (III) chloride/ascorbate, and hematin
Oxidation (nmoles MDA/substrate/hr)
0
100
200
300
400
Linoleic Acid (1mM)
Phospholipid (10mM)
LDL (1mM PL)
SLO (5000 units)
FeCl3/Ascorbate (50μM
Hematin (50μM)

Figure 4. The effects of soybean lipoxygenase oxidation on linoleic acid micelles. Linoleic acid micelles (0.5mM) were oxidized with SLO at room temperature at the indicated concentration. SLO was added to micelles in 0.2M BTP buffer (pH 7.4-9.5). Conjugated diene formation of the fatty acid was monitored during linoleic acid oxidation through UV absorbance readings at 234 nm taken every 10 seconds for 4 minutes. The extent of oxidation was calculated as a change in A_{234}/min. Initiated oxidation of SLO was compared to that of auto-oxidation (control). Data points represent the mean values of triplicate samples with SE < 2%.

The effects of soybean lipoxygenase oxidation on linoleic acid micelles

Figure 5. Effects of ketoconazole (KC) on oxidation of 0.5mM linoleic acid by SLO. Linoleic acid micelles (in BTP buffer) were oxidized with SLO at the indicated concentration at room temperature. SLO and KC (0-100μM) were added to micelles in 0.2M BTP buffer (pH 7.4 and 8.6). Conjugated diene formation of the fatty acid was monitored during linoleic acid oxidation through UV absorbance readings at 234 nm taken every 10 seconds for 4 minutes. Control (auto-oxidation) samples contained no SLO or KC. Data points represent the mean values of triplicate samples with SE < 2.2%.

Effects of ketoconazole (KC) on oxidation of (0.5mM) linoleic acid by SLO



pharmacological levels (>25μM), KC was able to reduce oxidation greater than 50% at pH 8.6.

On the other hand, as seen in figure 6, KC at dosages equal to or less than 25μM was able to attenuate linoleic acid oxidation by SLO (1000 units), hematin (5μM), and iron (III) chloride/ascorbate (10μM, 1:1) at pH 7.4. Hematin and SLO initiated oxidation of linoleic acid at physiological pH was lowered to the level of control (auto-oxidation) by KC at 25μM while $FeCl_3$/ascorbate induced oxidation of linoleic acid was reduced to 50% of the non-drug treated samples.

Phospholipid liposomes have been previously shown to be susceptible to oxidative attack by iron (III) chloride/ascorbate (Wiseman, 1991). The experiments represented in Figures 7 and 8 reconfirmed this (with 50μM $FeCl_3$/ascorbate) and showed that the liposomes were able to be oxidatively modified by SLO (5000 units) and hematin (50μM), although the free iron was comparatively the strongest initiator. In these experiments 2.5 mg/ml of phospholipid was used for oxidative purposes and the antioxidant effects of KC were observed.

Figure 7 demonstrated that 25μM KC in free (nonincorporated) form was able to reduce oxidation of phospholipid liposomes at or greater than 50% of initiated oxidation by SLO, FeCl3/ascorbate, and hematin. This attenuation continued as the concentration of KC was increased to 100μM. Furthermore, the data presented in figure 8 clearly shows a stronger antioxidant effect of KC when the drug was directly incorporated into the liposomes before they were exposed to oxidative attack by the various initiators. Incorporated on a molar basis with phospholipid, KC was able to inhibit phospholipid oxidation more effectively and at much lower dosages (10^{-4} moles ketoconazole:phospholipid (KC:PL) incorporated into the liposome is equivalent to a 0.1μM dose of KC in unincorporated form).

Figure 6. Effects of ketoconazole on linoleic acid micelle oxidation. Linoleic acid micelles (0.5mM) were oxidized with initiators SLO, hematin, and $FeCl_3$/ascorbate at the indicated concentrations. The initiators and KC (0-25μM) were added to micelles in PBS, pH 7.4. After 3 minutes at 37°C, the reaction was terminated and assayed for TBARS as described in Materials and Methods. Control samples contained no initiators or KC. Data points represent the mean values triplicate samples withn SE < 6%.

Effects of ketoconazole on linoleic acid micelle oxidation

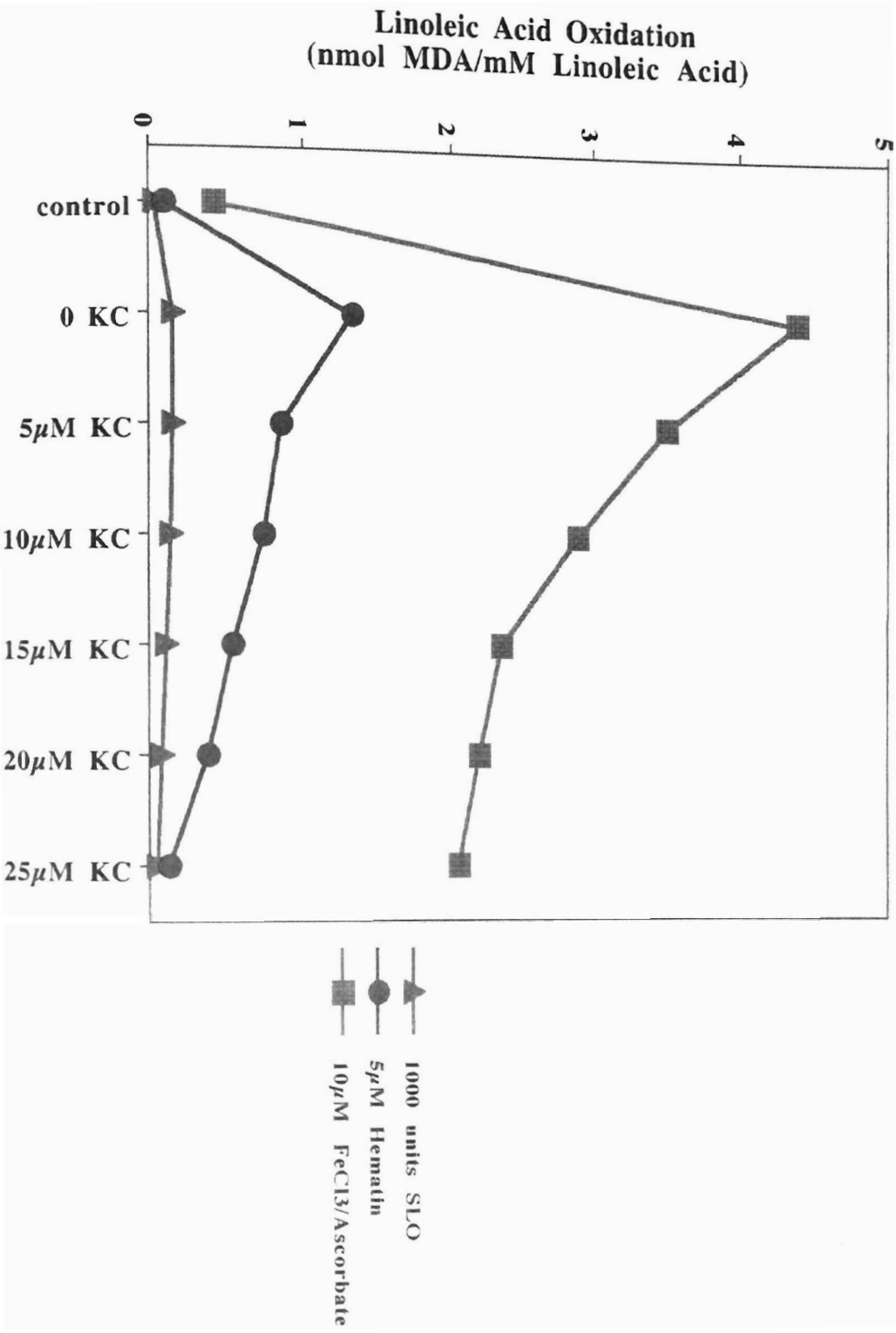


Figure 7. Effects of ketoconazole on oxidation of ox brain phospholipid liposomes. Phospholipid liposomes (2.5mg/ml) were oxidized with initiators SLO, hematin, and $FeCl_3$/ascorbate at the indicated concentrations. The initiators and KC (0-100μM) were added to liposomes in PBS, pH 7.4. After 20 minutes at 37°C, the reaction was terminated and assayed for TBARS as described in the Materials and Methods. Control samples contained no initiators or KC. Data points represent the mean values of triplicate samples with SE < 6.2%.

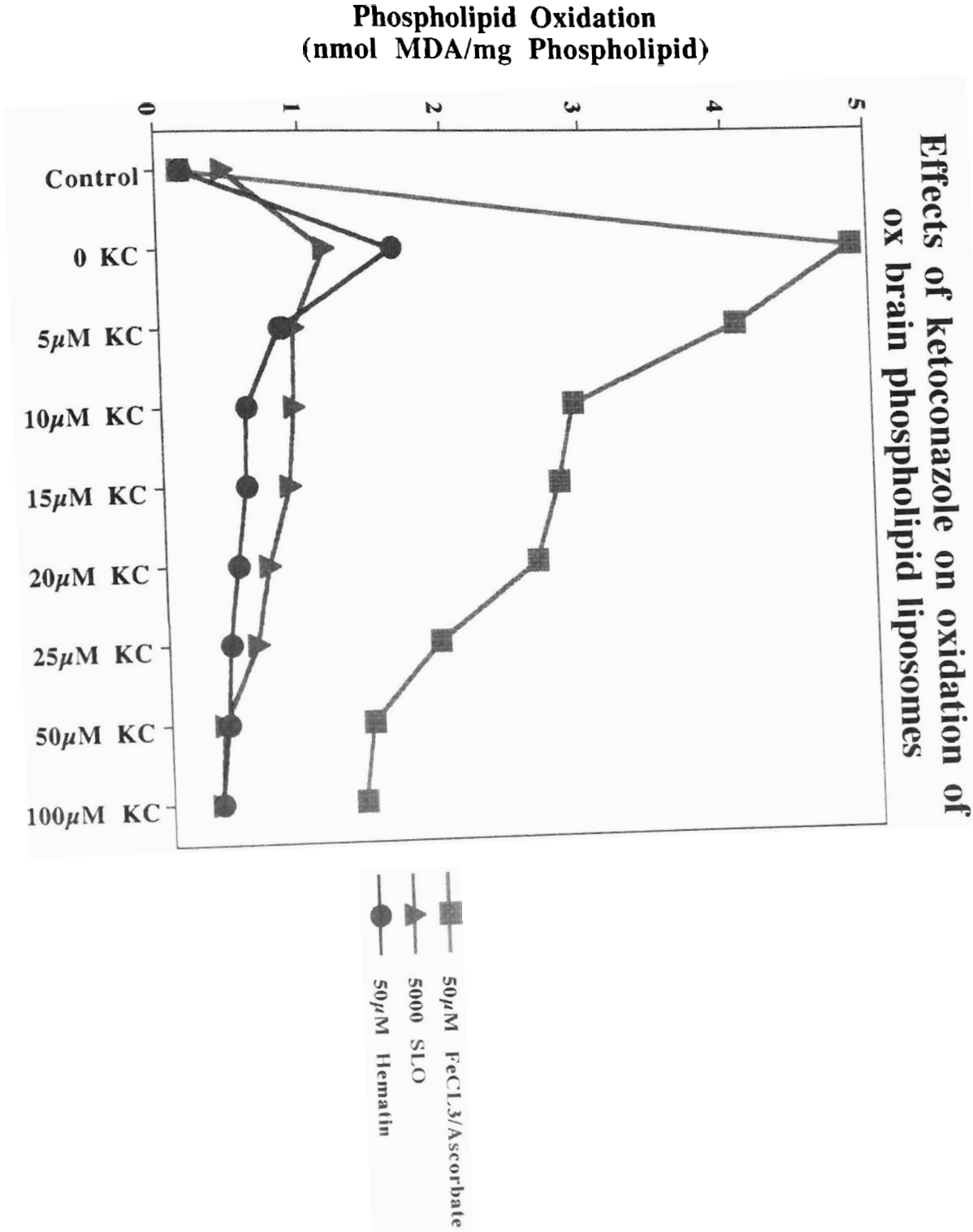

Effects of ketoconazole on oxidation of ox brain phospholipid liposomes
Phospholipid Oxidation
(nmol MDA/mg Phospholipid)
0
1
2
3
4
5
Control
0 KC
5μM KC
10μM KC
15μM KC
20μM KC
25μM KC
50μM KC
100μM KC
50μM FeCL3/Ascorbate
5000 SLO
50μM Hematin

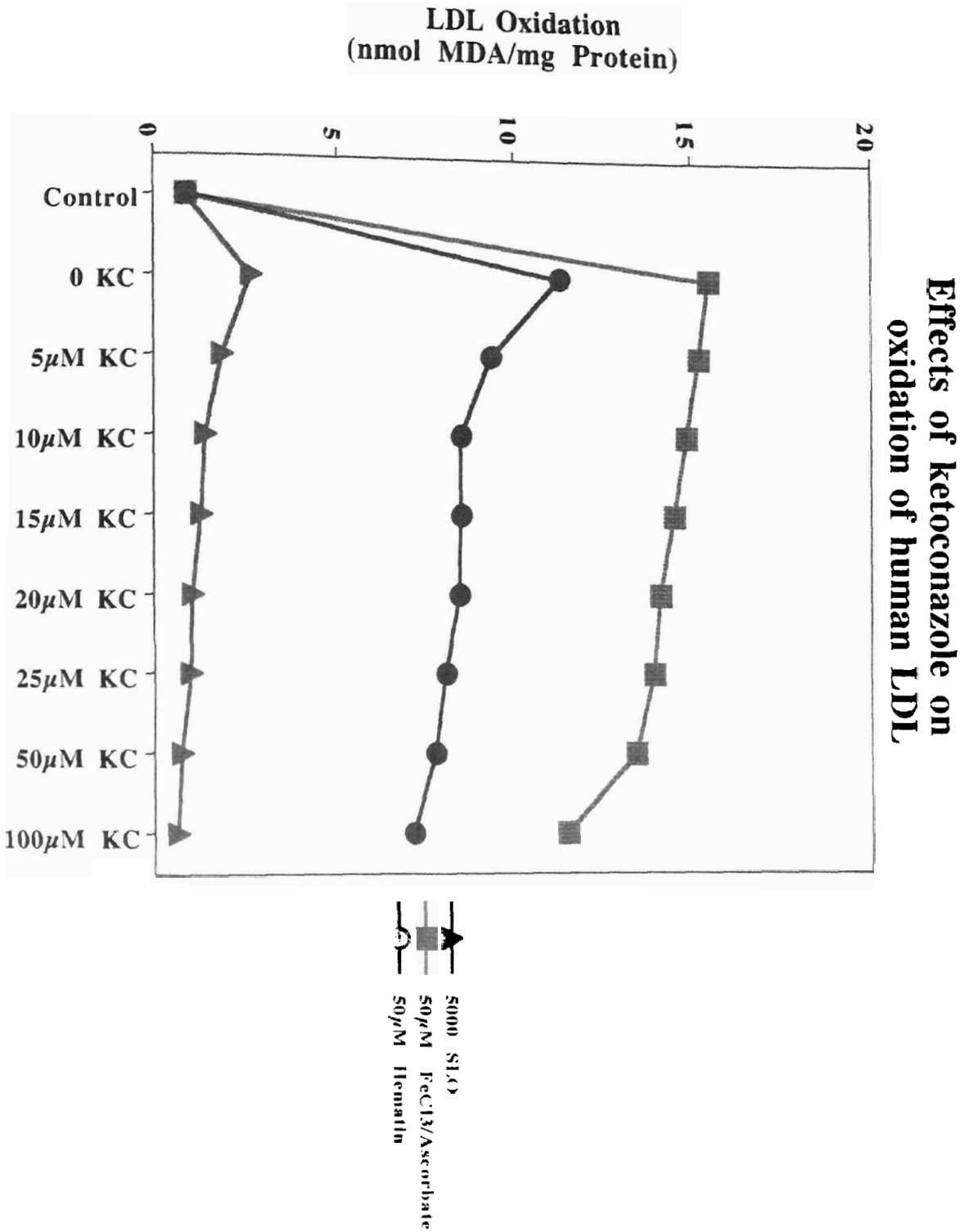
Figure 8. Effects of incorporated ketoconazole on oxidation of ox brain phospholipid liposomes. Phospholipid liposomes (2.5 mg/ml) were oxidized with initiators SLO, FeCl3/ascorbate, and hematin at the indicated concentrations. KC was incorporated into the liposomes prior to oxidation on a mole to mole basis of phospholipid content. Initiators were added to liposomes in PBS, pH 7.4. After 20 minutes at 37°C, the reaction was terminated and assayed for TBARS as described in Materials and Methods. Control samples contained no initiators or KC. Data points represent the mean values of triplicate samples with SE < 3%.

Effects of incorporated ketoconazole on oxidation of ox brain phospholipid liposomes
Phospholipid Oxidation (nmol MDA/mg Phospholipid)
0
1
2
3
4
5
6
Control
0 KC
1e-4m KC:PL
1e-3m KC:PL
1e-2m KC:PL
1e-1m KC:PL
50μM FeCl3/Ascorbat
5000 units SLO
50μM Hematin

Hematin's oxidative action on phospholipid liposomes was almost completely arrested when 10^{-4} moles KC:PL was first incorporated into the liposome. Any further incorporation of KC into the liposome revealed no additional protection. SLO was inhibited by 50% at approximately 10^{-4} moles KC:PL incorporation as compared to a dosage of 20μM KC in unincorporated form (figure 7). Complete inhibition was achieved by 10^{-1} moles KC:PL incorporation (100μM free KC). As with unincorporated KC, incorporated KC into the liposome attenuated liposome oxidation by free iron ($FeCl_3$/ascorbate) in a concentration dependent manner. Again, incorporated KC was more effective at lower dosages than unincorporated KC. Incorporation of KC into the liposome prior to liposome oxidation revealed a ten fold decrease in the dosage required to produce the same inhibitory effects as nonincorporated KC.

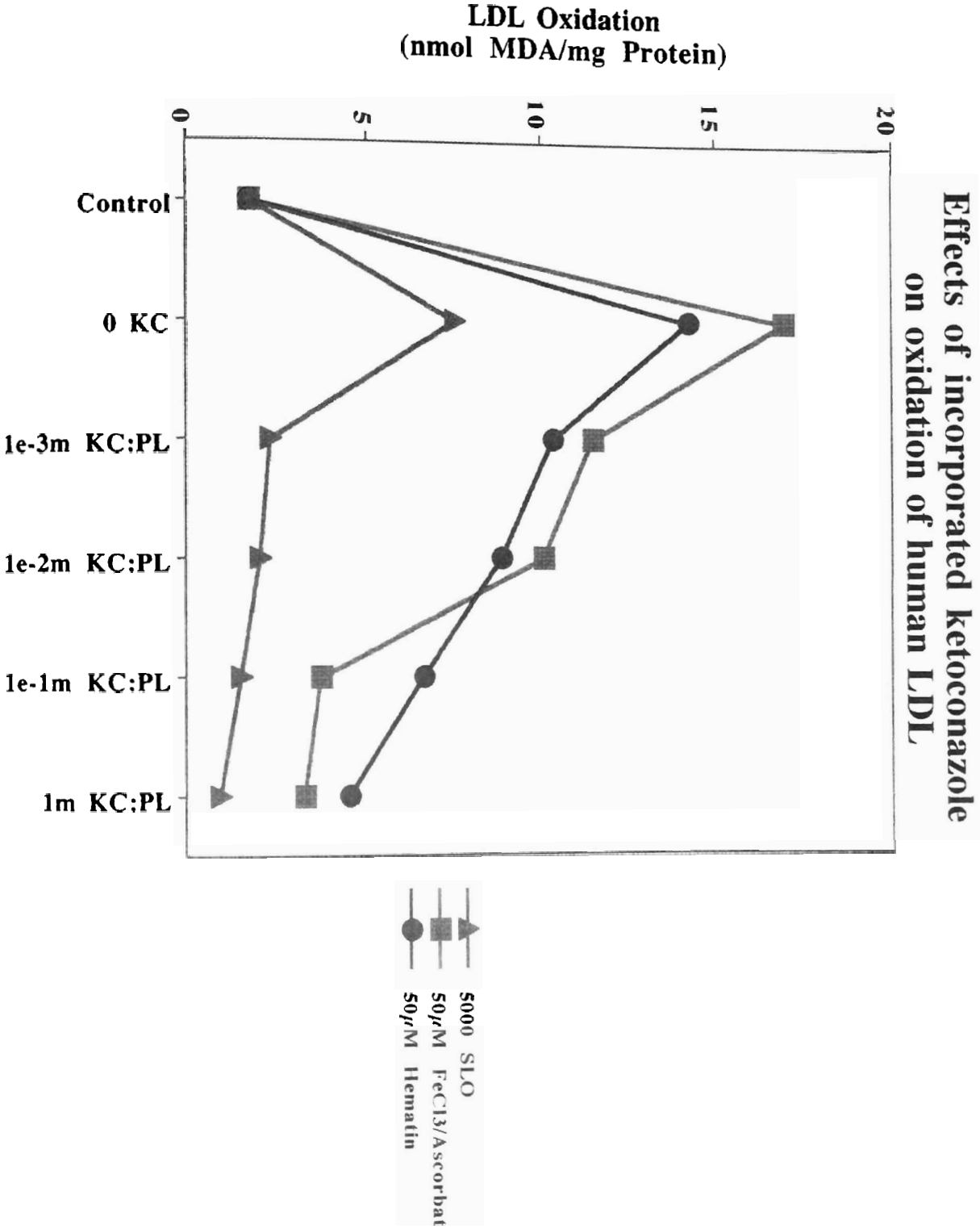
A greater difference between the effects of unincorporated versus incorporated KC on the oxidation of LDL was demonstrated in figures 9 and 10. It was shown in figure 9 that while LDL oxidation by SLO was inhibited by nonincorporated KC, the oxidative effects of free iron and hematin on LDL were not greatly reduced. However, as **figure 10** clearly demonstrates, when KC is incorporated into LDL particles as a pretreatment, inhibition of lipid oxidation by the various initiators was both concentration dependent and greater than 50%. SLO's action on LDL was completely attenuated when 10^{-3} moles KC:PL was incorporated into LDL (equivalent to 1μM free KC--see figure 9). As the amount of KC incorporated into LDL was increased, greater attenuation was achieved below the level of auto-oxidation. Oxidation of LDL by hematin was reduced 50% by pretreating LDL with 10^{-2} moles KC:PL (equivalent to 10μM free KC). Inhibition continued as more KC was incorporated. Free iron's action on LDL was equally inhibited by incorporated KC, showing 50% inhibition with 10^{-2} moles KC:PL incorporation and 66% inhibition when 1 mole KC:PL was incorporated into LDL.

Figure 9. Effects of ketoconazole on oxidation of human LDL. Isolated human LDL (0.5 mg protein/ml) was oxidized with initiators SLO, hematin, and $FeCl_3$/ascorbate at the indicated concentrations. The initiators and KC (0-100μM) were added to LDL in PBS, pH7.4. After 24 hours at 37°C, the reaction was terminated and assayed for TBARS as described in the Materials and Methods section. Control samples contained no initiators or KC. Data points represent the mean values of triplicate samples with SE < 5%



Effects of ketoconazole on oxidation of human LDL

Figure 10. Effects of incorporated ketoconazole on oxidation of human LDL. Isolated human LDL (0.5 mg protein/ml) was oxidized with initiators SLO, hematin, and $FeCl_3$/ascorbate at the indicated concentrations. KC was incorporated into LDL prior to oxidation on a mole to mole basis of phospholipid content. Initiators were added to LDL in PBS, pH 7.4. After 24 hours at 37°C, the reaction was terminated and assayed for TBARS as described in Materials and Methods. Control samples contained no initiators or KC. Data points represent the mean values of triplicate samples with SE < 8%.


Effects of incorporated ketoconazole on oxidation of human LDL
LDL Oxidation (nmol MDA/mg Protein)
0
5
10
15
20
Control
0 KC
1e-3m KC:PL
1e-2m KC:PL
1e-1m KC:PL
1m KC:PL
5000 SLO
50μM FeCl3/Ascorbat
50μM Hematin

DISCUSSION

The results presented above, in addition to what is already known about the structure and reactive nature of linoleic acid micelles, phospholipid liposomes, and LDL, allows for a more complete picture of the ability of iron moieties to oxidatively modify LDL and its model components. They also allow for theoretical conclusions concerning the way in which ketoconazole is able to attenuate such oxidation.

Within the lipid model system, linoleic acid can be considered a component of the inner LDL core within the triacylglycerols. Linoleic acid micelles, being unilamellar particles of free fatty acid (figure 2), are readily oxidized (figures 3-6). They are converted into conjugated dienes and, among other compounds, aldehyde products, malondialdehyde being prominent among them (Dix and Marnett 1985). The data (figures 3-6) suggest that, of the three iron moieties studied (heme, SLO, and free iron), free iron in the form of $FeCl_3$/ascorbate is the strongest initiator of oxidation in micelles because it is not bound to anything and therefore can easily fit into the lipid membrane. The iron in hematin, being bound similarly to heme, is a successful initiator, but not to the same extent as free iron. SLO is not near as successful as an oxidative initiator as hematin and free iron. However, as the pH is raised from physiological pH to a level between pH 8.5 and 9.0, the reactivity of SLO with linoleic acid micelles reaches an optimum (figures 4,5), suggesting that optimal surroundings for oxidative modification of lipids by SLO is at a higher pH than physiological.

Phospholipid liposomes, due to their multilamellar nature (figure 2) are more stable than the unilamellar linoleic acid micelles. They require a greater length of time to produce comparable oxidative products (figure 3). Because phospholipid creates a monolayer around the LDL particle, the phospholipid liposome with its outer boundary built of a phospholipid monolayer is an ideal model system for studying the effects of oxidative

attack to the outer shell of the lipid membrane. While liposomes are fairly stable, free iron can penetrate the lipid membrane and cause oxidative modification, as indicated by the increase in TBAR values (figures 3, 7, 8). The iron moieties of hematin and SLO, being bound and therefore cumbersome, are not able to penetrate the liposome as easily However, prevention is not complete as the liposome is constructed of external hydrophilic heads followed by an internal hydrophobic carbon chain similar to that of the linoleic acid micelle (figure 2).

While LDL is not as readily oxidized as linoleic acid due to its more complex structure (figure 2), it is also not as stable as phospholipid liposomes for the same reason. Once again, free iron is the strongest initiator of those studied. Hematin, while not as strong with its bound iron, is still able to penetrate the unwieldy outer layer of the LDL particle to reach the inner core. This phenomenon may also be due to the free (unesterified) cholesterol found along the outside of the LDL particle with phospholipid monolayer. It may be that disrupting the cholesterol molecules may be enough to disrupt the fluidity of the membrane, allowing the hematin to react with the outer phospholipid and inner triacylglycerol components. SLO continues to be the weakest initiator (figures 3, 9, 10), partly because it is not at optimum pH and partly due to the bound iron moiety that is not allowed to react in the manner of hematin.

While ketoconazole is already a successful antifungal drug, it has shown potential as an antioxidant (Wiseman et al. 1991). Ketoconazole was able to inhibit oxidation by all three initiators in all three lipid sources. In linoleic acid, ketoconazole was able to inhibit the oxidation of SLO at both physiological (pH 7.4) and optimal (pH 8.5-9.0) pH for the enzyme (figures 5, 6). This was true within the cellular level range ($\leq 25\mu M$) (Cathcart et al. 1991 and Eve Zentrich, unpubl.) and enhanced at pharmacological levels ($>25\mu M$). Hematin and free iron oxidation was attenuated at physiological pH within the cellular level range to at 50% (figure 6). These results indicate that ketoconazole is multifunctional. It is

possible that the iron moieties of the initiators are being surrounded (chelated) by ketoconazole to prevent them from interacting with the lipid membrane. It is also likely that the drug is directly interacting with the lipid membrane itself to protect it from oxidative attack.

It is suggested by the effect of ketoconazole on the oxidation of phospholipid liposomes and LDL that the greater protective action of ketoconazole is its direct association with the lipid membrane (figures 7-10). As illustrated in figures 7 and 8, ketoconazole is more effective against oxidative attack at lower doses than free KC if it is first incorporated directly into the liposome. Free (unincorporated) ketoconazole is able to attenuate the oxidation of the initiators equal to or greater than 50% (figure 7). Another line of evidence suggesting ketoconazole's protective effect on the membrane of liposome is the fact that smaller concentrations of free ketoconazole are able to attenuate oxidative attack by relatively higher concentrations of the three initiators. It would seem unlikely that such a small amount of drug would be able to chelate such a large amount of iron moiety (figures 7 and 8).

This apparent antioxidant action of ketoconazole is further demonstrated in its action upon LDL (figures 9 and 10). LDL oxidation by SLO is easily inhibited by free (unincorporated) ketoconazole. Ketoconazole either directly protects the particle membrane from oxidative attack and/or it blocks the iron moiety of the enzyme. The modifying actions of hematin and free iron on LDL are not easily attenuated by free KC (figure 9). However, when LDL is pretreated with ketoconazole so that the drug is incorporated directly into the lipid membrane, only micromolar amounts of ketoconazole are required to block the action of hematin and free iron. Due to the concentration-dependent manner in which attenuation of oxidation is achieved by incorporated ketoconazole, it is possible that the modifying action hematin and free iron on LDL can be totally blocked with the proper pharmacological dosage of KC (figure 10).

It is suggested by this study that ketoconazole, as an antioxidant, acts primarily to protect the lipid membrane from oxidative attack and perhaps has a minor role in chelating iron moieties. Future studies involving spectroflurometry (inserting a tumbling flur into the lipid membrane to look at its fluid properties) could shed more light on this matter. Clinical studies considering the effect of ketoconazole on patients with hypercholesterolemia show a significant decrease in the plasma levels of cholesterol precursors and LDL (Gylling et al. 1993), further suggesting that ketoconazole may be an already existing drug that can limit the adverse effects of atherosclerosis.

CONCLUSIONS

1. Linoleic acid micelles, phospholipid liposomes, and LDL were oxidized by the initiators SLO, free iron, and hematin (figure 3). Linoleic acid micelles were oxidized by SLO at its optimum pH, pH 8.5-9.0 (figure 4).

2. Free (unincorporated) KC, at 25-100μM, attenuated lipid oxidation (figures 6, 7, 9). Free KC also attenuated linoleic oxidation by SLO at pH 8.6 (figure 5).

3. Oxidation of phospholipid liposomes and LDL by the various initiators was inhibited by KC more effectively if KC was incorporated into the lipid substrate prior to oxidative attack (figures 8, 10) .

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